

Dual requirement for yeast hnRNP Nab2p in mRNA poly(A) tail length control and nuclear export

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Recent studies of mRNA export factors have provided additional evidence for a mechanistic link between mRNA 3'-end formation and nuclear export. Here, we identify Nab2p as a nuclear poly(A)-binding protein required for both poly(A) tail length control and nuclear export of mRNA. Loss of *NAB2* expression leads to hyperadenylation and nuclear accumulation of poly(A)⁺ RNA but, in contrast to mRNA export mutants, these defects can be uncoupled in a *nab2* mutant strain. Previous studies have implicated the cytoplasmic poly(A) tail-binding protein Pab1p in poly(A) tail length control during polyadenylation. Although cells are viable in the absence of *NAB2* expression when *PAB1* is overexpressed, Pab1p fails to resolve the *nab2Δ* hyperadenylation defect even when Pab1p is tagged with a nuclear localization sequence and targeted to the nucleus. These results indicate that Nab2p is essential for poly(A) tail length control *in vivo*, and we demonstrate that Nab2p activates polyadenylation, while inhibiting hyperadenylation, in the absence of Pab1p *in vitro*. We propose that Nab2p provides an important link between the termination of mRNA polyadenylation and nuclear export.

Keywords: hnRNP/mRNA export/nuclear poly(A)-binding protein/polyadenylation

Introduction

The majority of RNA polymerase II transcripts are modified by 3'-end endonucleolytic cleavage and polyadenylation (for reviews see Minvielle-Sebastia and Keller, 1999; Zhao *et al.*, 1999). In mammals, poly(A) tails reach a maximum of 200–300 nucleotides, and the nuclear poly(A) tail-binding protein PABPN1 (formerly PABP2) is essential for regulating the efficiency and extent of polyadenylation. The PABPN1 protein binds to

nascent oligo(A) tails during pre-mRNA 3'-end processing and interacts with the multisubunit cleavage and polyadenylation specificity factor (CPSF) and poly(A) polymerase to promote rapid formation of the extended poly(A) tail (Wahle, 1991, 1995). When the tail reaches 200–300 nucleotides, the processive phase of the polyadenylation reaction terminates (Wahle, 1995; Keller *et al.*, 2000).

In *Saccharomyces cerevisiae*, poly(A) tails are considerably shorter (70–90 nucleotides), and a PABPN1 ortholog does not exist. Instead, the cytoplasmic poly(A) tail-binding protein Pab1p (PABP1 in metazoans) has been proposed to coat the poly(A) tail during polyadenylation to restrict tail length in a manner analogous to PABPN1 (Zhao *et al.*, 1999). Indeed, human PABP1 shuttles between the nucleus and cytoplasm (Afonina *et al.*, 1998), suggesting that this predominantly cytoplasmic protein also functions in the nucleus. The Pab1 protein is associated with cleavage factor IA (CF IA), which is involved in both the cleavage and polyadenylation steps, and Pab1p is required for poly(A) tail length regulation *in vitro* (Amrani *et al.*, 1997; Minvielle-Sebastia *et al.*, 1997). An alternative to this model is that long tails may be synthesized initially, and then shortened by a poly(A) nuclease (PAN) activity that is recruited by Pab1p bound to poly(A) (Brown and Sachs, 1998). In support of this latter possibility, poly(A) tails >200 nucleotides are added to a cytochrome *c* (CYC1) precursor RNA *in vitro* using extracts from *pan* mutant strains. However, several observations argue against the proposal that Pab1p is the sole nuclear poly(A) tail-binding protein that regulates tail length by coating the poly(A) tail. First, the steady-state concentration of Pab1p in the nucleus is low so it must compete with more abundant nuclear RNA-binding proteins for poly(A) tail binding (Anderson *et al.*, 1993a). Secondly, the very long tails produced *in vitro* in the absence of Pab1p are not detectable in either *pab1* or *pan* mutant strains *in vivo*, which show tails only 3–20 nucleotides longer (Sachs and Davis, 1989; Brown and Sachs, 1998). Why this difference in maximum tail length *in vitro* and *in vivo*? Tail length may be restricted in the nucleus by competition between different transcripts for 3'-end processing factors, or termination of polyadenylation might be induced by ongoing nuclear export (Brown and Sachs, 1998). Alternatively, a tail length regulatory factor might be lost, or inactivated, during preparation of the extracts used for mRNA 3'-end formation *in vitro*.

Following transcript maturation, nuclear mRNA–hnRNP complexes are exported into the cytoplasm through the nuclear pore complex (NPC) (for reviews see Cole, 2000; Cullen, 2000). Specialized interactions between mRNA-binding proteins and NPC-associated factors are critical for mRNA export (Rodrigues *et al.*, 2001). For example, the yeast protein Yra1p binds

preferentially to mature mRNA in the nucleus (Sträßer and Hurt, 2000). In turn, Yra1p is recognized by the heterodimer Mex67p–Mtr2p, which also associates with nucleoporins to facilitate nuclear mRNA export (Santos-Rosa *et al.*, 1998; Hurt *et al.*, 2000; Sträßer *et al.*, 2000). During, and/or following, mRNA export through the NPC, the major class of nuclear pre-mRNA/mRNA-binding proteins, the hnRNPs, dissociate from the mRNA and are recycled back into the nucleus by members of the importin/karyopherin β family.

Previous work has suggested that polyadenylation is also important for mRNA export (Eckner *et al.*, 1991; Huang and Carmichael, 1996). Indeed, recent studies on one class of mRNA export factor support the possibility that these two processes are mechanistically coupled (Hilleren and Parker, 2001; Hilleren *et al.*, 2001; Jensen *et al.*, 2001). What is the link between poly(A) tail addition and export? One model suggests that some mutations in mRNA export factors impair the release of nascent mRNA from its transcriptional site and lead to prolongation of the polyadenylation phase, possibly by depleting a 3'-end processing release/termination factor. In this report, we provide evidence that the yeast hnRNP Nab2p plays dual roles in the termination of mRNA polyadenylation and nuclear export.

Results

Hyperadenylated mRNAs accumulate in the nucleus following Nab2p depletion

Current models for the regulation of poly(A) tail length during mRNA 3'-end formation suggest that Pab1p coats the poly(A) tail during polyadenylation (Zhao *et al.*, 1999). Because Pab1p is not a major protein in the nucleus, we speculated that additional and more abundant nuclear poly(A)-binding proteins might function in polyadenylation. A candidate nuclear poly(A) tail-binding protein is Nab2p, which binds poly(A) *in vitro* and also appears to play a role in mRNA export (Anderson *et al.*, 1993b; Wilson *et al.*, 1994; Green *et al.*, 2002). To determine if Nab2p and Pab1p possessed similar binding affinities for poly(A), we employed a standard nitrocellulose filter binding assay. Previous work has shown that Pab1p occupies a binding site of ~12 nucleotides and human PABP1 binds tightly to (rA)₂₅ ($K_d = 7$ nM) (Görlach *et al.*, 1994). Interestingly, yeast Nab2p showed a similar (rA)₂₅ binding affinity (Figure 1A, $K_d = 10.5$ nM).

Since Nab2p and Pab1p bound to poly(A) with similar affinities *in vitro*, we investigated whether Nab2p was required for poly(A) tail length control *in vivo*. The *NAB2* gene was cloned downstream of the *GAL1* promoter on a low-copy plasmid to create pGAL::NAB2. The YJA517-1C haploid strain (see Table I), which carries both a *nab2Δ* chromosomal deletion and the pGAL::NAB2 plasmid, was viable on galactose but failed to grow on glucose (Figure 1B). Immunoblot analysis demonstrated that Nab2p depletion occurred slowly following repression of *NAB2* expression, and that growth was compromised following ~80% loss of Nab2p at 8 h (Figure 1C). Although no significant defects in either pre-mRNA splicing or pre-rRNA processing were detectable during this time period (data not shown), a striking effect on polyadenylation was observed. As early as 2 h following

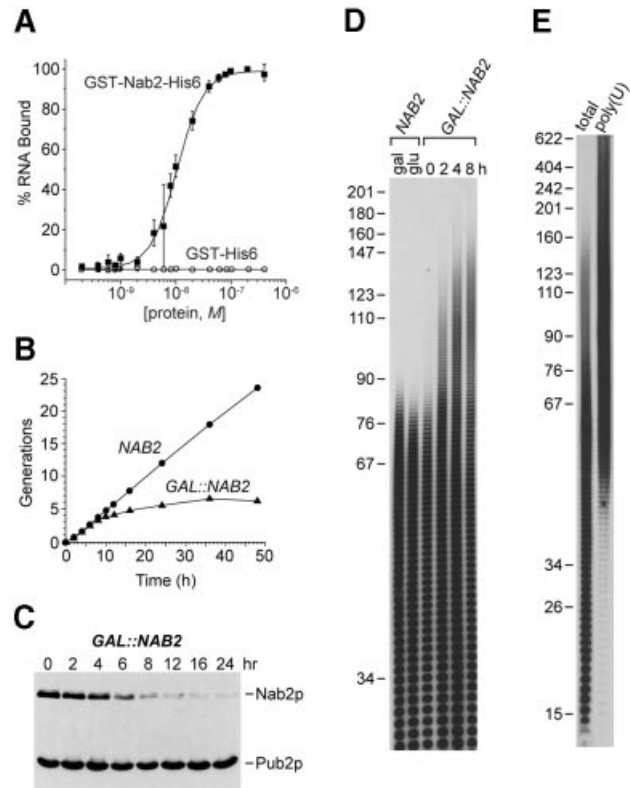


Fig. 1. Nab2p depletion leads to hyperadenylation of mRNA. (A) High affinity binding of Nab2p to poly(A). Nitrocellulose filter binding assays were performed using (rA)₂₅ and either GST–Nab2–His₆ (closed squares) or GST–His₆ (open circles). (B) Generation plot showing growth inhibition of YJA517-1C (*GAL::NAB2*) following glucose shift (0 h). (C) Immunoblot analysis using mAb 3F2 (anti-Nab2p) and mAb 3B1 (anti-Pub2p). YJA517-1C cells, grown in galactose, were shifted into glucose for 0–24 h. (D) Poly(A) tail analysis of *NAB2* versus *GAL::NAB2* cells grown in either galactose (gal) or glucose (glu) for 0–8 h. Each lane represents equal amounts of labeled RNA. The indicated size markers are pBR322 *Msp*I fragments. (E) Selection of hyperadenylated RNAs following Nab2p depletion *in vivo*. Poly(A) tail lengths were determined for total RNA isolated from YJA517-1C cells grown in glucose for 16 h (total) or following selection of hyperadenylated RNA from this total pool by poly(U)–Sephadex chromatography [poly(U)].

glucose-induced repression, when intracellular Nab2p levels had declined <2-fold, maximum poly(A) tail length rose dramatically, and by 8 h maximum tail length had increased from ~80 to >150 nucleotides (Figure 1D). This increase of >70 nucleotides was not due simply to alteration of the carbon source since cells that expressed wild-type Nab2p levels possessed normal poly(A) tail lengths when cultured in either glucose or galactose. Because non-mRNA RNA polymerase II transcripts are also polyadenylated under certain conditions (Chapon *et al.*, 1997; Abou Elela and Ares, 1998; van Hoof *et al.*, 2000), hyperpolyadenylated RNAs produced following Nab2p depletion were isolated selectively by poly(U)–Sephadex chromatography at an elevated formamide concentration (Figure 1E). These RNAs were identified by cDNA library construction followed by DNA sequencing of 100 cDNA clones. Whereas a variety of mRNAs were present in this long poly(A) tail pool, no snRNAs, snoRNAs or telomerase RNA were identified (our unpub-

Table I. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Reference
YJA512	<i>MATa</i> α <i>leu2Δ2/leu2Δ2 ura3-52/ura3-52 NAB2/nab2::LEU2</i> pNAB2.15	Anderson <i>et al.</i> (1993b)
YJA513	<i>MATa</i> <i>leu2Δ2 ura3-52 nab2Δ::LEU2</i> pNAB2.15	this study
YJA515	<i>MATa</i> <i>leu2Δ2 ura3-52 nab2Δ::LEU2</i> pNAB2.19	this study
YJA517	<i>MATa/MATa</i> <i>leu2Δ2/leu2Δ2 ura3-52/ura3-52 NAB2/nab2Δ::LEU2</i> pGAL::NAB2	this study
YJA517-1C	<i>MATa</i> <i>leu2Δ2 ura3-52 nab2Δ::LEU2</i> pGAL::NAB2	this study
YJA517-1D	<i>MATa</i> <i>leu2Δ2 ura3-52 NAB2</i>	this study
YJA221	<i>MATa</i> <i>leu2Δ2 his3Δ2 trp1-189 ura3-52 nab2Δ::LEU2</i> pnab2-21	this study
YJA223	<i>MATa</i> <i>leu2Δ2 his3Δ200 trp1-189 ura3-52 nab2Δ::LEU2</i> TRP1::nab2-21	this study
L4717	<i>MATa</i> <i>ade2 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>	Hong <i>et al.</i> (1997)
YKN105	<i>MATa</i> <i>ade2 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 nab2Δ::HIS3</i> pPAB1.6	this study
YKN106	<i>MATa</i> <i>ade2 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 nab2Δ::HIS3</i> pPAB1.7	this study
YRH201C	<i>MATa</i> <i>ade2 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 nab2Δ::HIS3</i> pGAL::NAB2	this study
YRH202	<i>MATa</i> <i>ade2 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 nab2Δ::HIS3</i> pGAL::NAB2 YEpl3	this study
YRH204	<i>MATa</i> <i>ade2 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 nab2Δ::HIS3</i> pPAB1	this study
YHCS220	<i>MATa</i> <i>ade2 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 nab2Δ::HIS3</i> pGAL::NAB2 pHCS220	this study
YAS394	<i>MATa</i> <i>pab1::HIS3 RPL46::LEU2 ade2 his3 leu2 trp1 ura3 can1</i>	Sachs and Deardorff (1992)
BMA64	<i>MATa</i> <i>ade2-1 leu2-3,112 ura3-1 trp1Δ his3-11,15 can1-100</i>	Baudin-Baillieu <i>et al.</i> (1997)
YSD10	<i>MATa</i> <i>TAP::FIP1-TRP1-K1 ade2-1 leu2-3,112 ura3-1 trp1Δ his3-11,15 can1-100</i>	S.Dheur and L.Minvielle-Sebastia (in preparation)

lished data). Therefore, Nab2p depletion leads to selective hyperadenylation of mRNA.

Some temperature-sensitive (ts) strains deficient in mRNA export at restrictive growth temperatures also accumulate hyperadenylated mRNAs when nuclear export is blocked (Kadowaki *et al.*, 1994; Tseng *et al.*, 1998; Hilleren and Parker, 2001; Jensen *et al.*, 2001). Thus, we examined the effect of depleting Nab2p on the intracellular distribution of poly(A)⁺ RNAs using fluorescence *in situ* hybridization (FISH). Cells expressing the wild-type *NAB2* gene during growth in either glucose or galactose possessed a disperse cytoplasmic poly(A)⁺ RNA staining pattern (Figure 2A). Although YJA517-1C (*GAL::NAB2*) cells also showed a normal poly(A)⁺ RNA distribution in galactose, when these cells were shifted to glucose, intense subnuclear regions, or foci, appeared within 2 h with a concomitant loss of cytoplasmic fluorescence signal (Figure 2A and B). Similarly to the mRNA transport-defective strain *mtr2-1* (Kadowaki *et al.*, 1994), these poly(A)⁺ RNA-rich foci co-localized with the nucleolar protein Nop1p (Figure 2C). These results supported the hypothesis that Nab2p was required for mRNA export and, similarly to other characterized mRNA export mutants, the hyperadenylated phenotype was due to an export block. To test this hypothesis, we created a conditional lethal *nab2* allele and examined whether the poly(A) tail and export phenotypes were linked obligatorily in this mutant.

Uncoupling of mRNA polyadenylation and export defects in a *nab2* mutant

A number of mRNA export mutants have been identified by screening randomly generated ts strains for accumulation of poly(A)⁺ RNA in the nucleus (Amberg *et al.*, 1992; Kadowaki *et al.*, 1994). These screens failed to identify *nab2* ts alleles. As an alternative, the cold-sensitive *nab2-21* allele was constructed by eliminating the *NAB2* sequence encoding amino acids 424–445. This 21 amino acid deletion includes part of the sixth, and all of the seventh, C₃H repeat that is required for binding to poly(A)

in vitro (Anderson *et al.*, 1993b). When tested for viability at different temperatures, the *nab2-21* strain grew similarly to wild-type strains at both 30 and 37°C, but was growth inhibited at 14°C (Figure 3A). Surprisingly, long poly(A) tails (maximum tail length ~130 nucleotides) were detected at both permissive (30°C) and restrictive (14°C) growth temperatures, demonstrating that hyperadenylation alone was not growth inhibitory (Figure 3B). In contrast, FISH analysis showed accumulation of poly(A)⁺ RNA in 1 or 2 foci per nucleus only at 14°C (Figure 3C). In agreement with the Nab2p depletion studies, poly(A)⁺ RNA accumulated in the nucleus and co-localized with Nop1p (Figure 3D). Since the poly(A) tail length and FISH analyses may not be equally sensitive, these observations did not eliminate the possibility that tail lengths increased at 14°C, or that mRNA export was slightly impaired at 30°C, in *nab2-21*. Nevertheless, our results were consistent with the hypothesis that Nab2p was required for poly(A) tail length regulation, but long poly(A) tails were not growth inhibitory. To examine this proposal further, we identified genes whose overexpression compensated for loss of *NAB2* function.

***PAB1* is a high-copy suppressor of *nab2* mutant alleles**

For the high copy suppressor screen, *GAL::NAB2* cells (YRH201C) were grown in galactose, transformed with a yeast genomic DNA library cloned into a multicopy plasmid and subsequently transferred to growth on glucose to repress *NAB2* transcription. Out of ~10⁶ transformants, 20 colonies grew in glucose following restreaking, and 19 of these were identified as carrying *NAB2* by PCR screening. The remaining strain (*nab2 Δ* pHCS220) did not carry a plasmid-borne *NAB2* copy but grew well on glucose (Figure 4A). Sequencing of the pHCS220 plasmid revealed that it contained a 10 kb genomic fragment that included the *CHD1*, *PAB1* and *DNF1* genes. All three genes were subcloned individually and tested for suppression. Only the *nab2 Δ* pPAB1 strain grew in glucose, suggesting that Pab1p overexpression compensated for

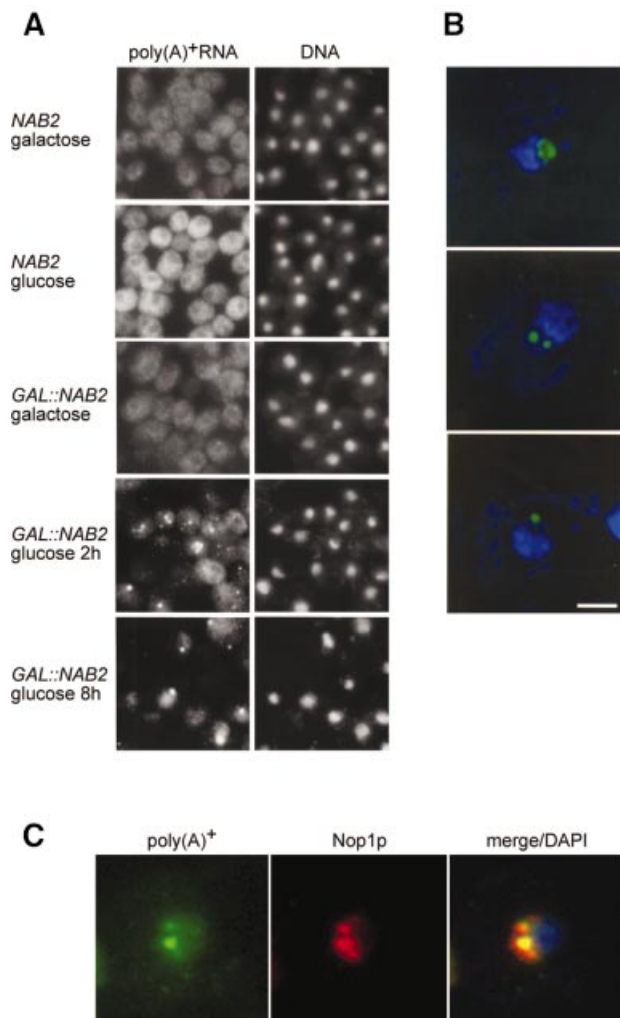


Fig. 2. Accumulation of poly(A)⁺ RNA in the nucleus and nucleolus following loss of Nab2p *in vivo*. **(A)** FISH analysis of poly(A)⁺ RNA distribution using conventional immunofluorescence microscopy. DNA was detected with DAPI. **(B)** FISH localization of poly(A)⁺ RNA using deconvolution microscopy. The distribution of poly(A)⁺ RNA (green) is shown for YJA517-1C (*GAL::NAB2*) cells shifted to glucose for 2 h. The nucleolus is adjacent to the bulk chromosomal DNA staining (blue). Three nucleolar distributions are displayed (top panel, overall nucleolar; middle panel, two intra-nucleolar foci; bottom panel, a single focus at the nucleolar periphery). Bar = 2 μ m. **(C)** Co-localization of poly(A)⁺ RNA (green) and Nop1p (red) in *GAL::NAB2* cells shifted to glucose for 2 h. Merged poly(A)⁺ RNA, Nop1p and DNA signals are shown in the right panel.

loss of Nab2p (Figure 4A), and this was confirmed by immunoblot analysis (Figure 4B). The intracellular level of the 70 kDa full-length Pab1p increased ~2-fold, while a 53 kDa Pab1p fragment (Figure 4B, Pab1p*) was not detectable in wild-type *NAB2* strains but was present in both *nab2Δ* pHCS220 and *nab2Δ* pPAB1 strains. The Pab1p* polypeptide was generated by proteolysis within the C-terminus of Pab1p since it was detectable with monoclonal antibody (mAb) 1G1, which recognizes an N-terminal epitope (our unpublished data). Surprisingly, long poly(A) tails were still detectable in *nab2Δ* pHCS220 and *nab2Δ* pPAB1 cells (Figure 4C). To confirm that long poly(A) tails were added to mRNAs in *nab2Δ* pPAB1 cells, poly(A) tail lengths were determined for several transcripts using the PCR-based poly(A) tail length (PAT)

assay (Salles *et al.*, 1999). All of the tested cDNAs that were identified originally in the hyperadenylated cDNA library (see Figure 1E) showed long poly(A) tails in this assay, including *PGK1* and *SED1* transcripts (Figure 4D). Therefore, mRNAs with long poly(A) tails were present in both *nab2-21* and *nab2Δ* pPAB1 cells under conditions permissive for growth.

An important question that emerged from these studies was the mechanism of *PAB1* suppression. *PAB1* is also a dose-dependent suppressor of *ma15-2* and, since Pab1p and Rna15p are CF I subunits, a previous study proposed that elevated nuclear levels of Pab1p compensate directly for loss of Rna15p activity in mRNA 3'-end formation (Amrani *et al.*, 1997). Since it was possible that over-expressed Pab1p also accumulated in the nucleus of *nab2Δ* cells and compensated for Nab2p loss, we examined the subcellular distribution of Pab1p in *nab2Δ* pPAB1 cells. In agreement with a previous study (Anderson *et al.*, 1993a), Pab1p was localized predominantly in the cytoplasm in wild-type cells while Npl3p and Nab2p were distributed in the nucleus (Figure 4E). Although the distribution of Npl3p was identical in the *nab2Δ* pHCS220 and *nab2Δ* pPAB1 strains that were devoid of Nab2p, overexpressed Pab1p was detectable throughout these cells. Deconvolution microscopy demonstrated that Pab1p was not detectable in the nuclei or vacuoles of *NAB2* wild-type cells, but was present in both of these compartments in *nab2Δ* pPAB1 cells (Figure 4E). Thus, *PAB1* over-expression in a *nab2Δ* background led to the appearance of Pab1p in the nucleus, and correlated with resolution of the *nab2Δ* growth defect. However, Pab1p levels were elevated throughout the cell so it was possible that an increase in the cytoplasmic level of Pab1p was responsible for viability of the *nab2Δ* pPAB1 strain. To determine if *PAB1* suppression required nuclear import of Pab1p, *nab2Δ* suppression analysis was performed using a modified *PAB1* allele on a low-copy plasmid that expressed a nuclear-targeted form of Pab1p.

Nuclear targeting of Pab1p suppresses the *nab2Δ* growth defect

We used plasmid shuffling to replace the multicopy pPAB1 plasmid in the *nab2Δ* pPAB1 strain with a CEN plasmid (pRS315) carrying a modified *PAB1* allele, which encoded Pab1p tagged with the SV40 nuclear localization (NL) sequence (PKKKRKV) at the N-terminus and a V5 epitope at the C-terminus. The *nab2Δ* pPAB1_{NL} strain was viable at 24°C (Figure 5A), and the Pab1 and Pab1_{NL} proteins were expressed at a similar level in *nab2Δ* pPAB1_{NL} cells (Figure 5B). Low-copy *PAB1* suppression of *nab2Δ* required nuclear targeting of Pab1p since *nab2Δ* *PAB1*_{mn1}, a strain carrying a *PAB1* allele with a non-functional (PKTKRKV) SV40 mutant NL (mn1) sequence (*nab2Δ* pPAB1_{mn1}), was not viable in glucose (Figure 5A) although the corresponding protein, Pab1_{mn1}, was expressed in galactose in the presence of pGAL::NAB2 (Figure 5B). As opposed to wild-type Pab1p, the Pab1_{NL} protein, visualized by cell immunofluorescence using the anti-V5 antibody, accumulated in the nucleus although this protein was also detectable in the cytoplasm (Figure 5C). Significantly, poly(A)⁺ RNA was detectable in both the nuclear and cytoplasmic compartments in the majority of cells expressing either pPAB1 or pPAB1_{NL}, suggesting

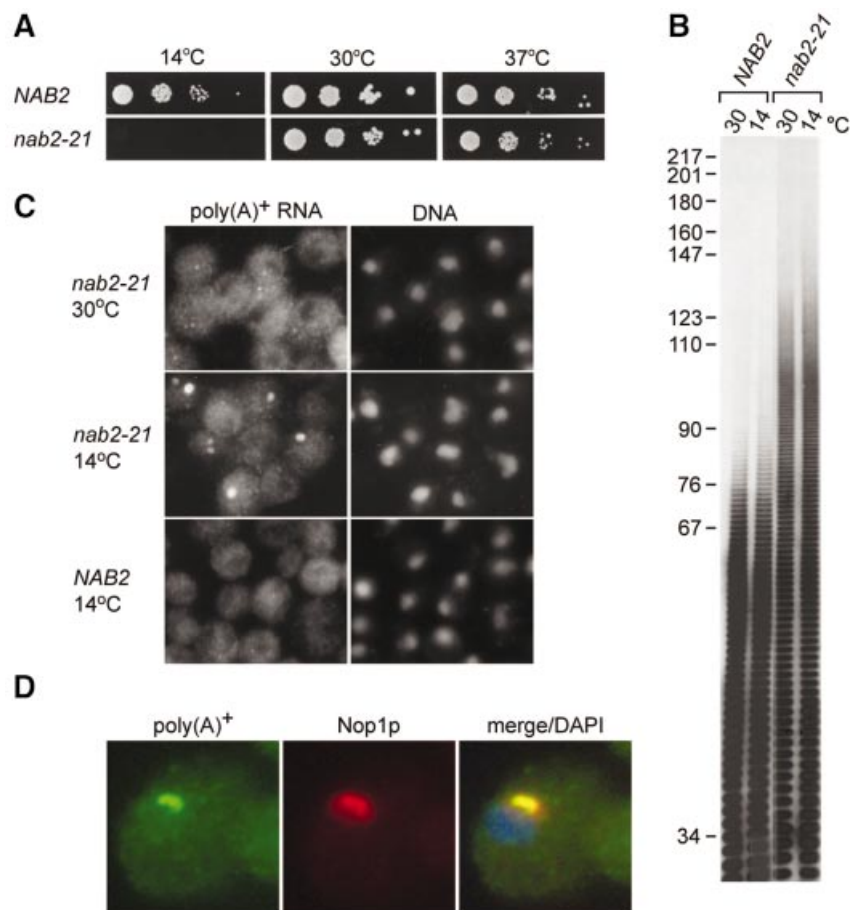


Fig. 3. Constitutive long poly(A) tails and inducible nuclear accumulation of poly(A)⁺ RNA in *nab2-21*. (A) Spot growth analysis of *NAB2* and *nab2-21* strains. Serial 10-fold dilutions of cells were plated on YPD plates. (B) Poly(A) tail analysis of *NAB2* and *nab2-21* at 30 and 14°C. (C) FISH analysis of poly(A)⁺ RNA distribution. (D) Poly(A)⁺ RNA accumulates in the nucleolus. The localization of poly(A)⁺ RNA (green) in *nab2-21* at 14°C is compared with the nucleolar protein Nop1p (red) and DNA (blue).

that cell viability was due to partial resolution of the *nab2Δ* mRNA export block by *PAB1* overexpression in these strains (compare Figures 5C and 2A). However, *nab2Δ* pPAB1_{NL} cells with the highest nuclear concentration of Pab1_{NL} protein also showed the most intense nuclear poly(A)⁺ RNA signals, which suggested that very high levels of Pab1p in the nucleus might impair mRNA export. In contrast to the effects on mRNA export and cell viability, pPAB1_{NL} expression exacerbated the *nab2Δ* hyperadenylation defect and maximum poly(A) tail lengths increased to ~10 nucleotides longer in *nab2Δ* pPAB1_{NL} as compared with *nab2Δ* pPAB1 cells (data not shown). On the basis of this latter observation, we speculated that Nab2p might be the primary factor that regulates poly(A) tail length *in vivo* by acting either as a polyadenylation termination, or a poly(A) tail trimming, factor. Loss of termination/trimming activity might delay mRNA entry into the export pool. To look at this possibility, we examined whether Nab2p was directly required for poly(A) tail length control during 3'-end processing *in vitro*.

Nab2p stimulates polyadenylation while restricting tail length *in vitro*

A potential role for Nab2p in mRNA 3'-end formation *in vitro* was examined using extracts from a *pab1Δ* strain

(YAS394). We used these extracts because preliminary studies using wild-type extracts indicated that Nab2p was not essential for CYC1 precursor RNA poly(A) tail length regulation (our unpublished data). However, the discovery that Pab1p is required for poly(A) tail length control *in vitro* suggested that the presence of Pab1p in these cell extracts might mask any role for Nab2p in polyadenylation (Amrani *et al.*, 1997; Minvielle-Sebastia *et al.*, 1997). In addition, Kap104p and Nab2p form a complex and Kap104p might bind to free Nab2p in the extract and prohibit its RNA-binding activity (Lee and Aitchison, 1999). In support of this possibility, we determined that Kap104p was present in the cell extracts used for polyadenylation, and the majority of Nab2p in these extracts could be immunodepleted with an anti-Kap104p mAb (data not shown). Therefore, *in vitro* 3'-end processing assays were performed with extracts from a *pab1Δ* strain in the absence or presence of recombinant Nab2p.

Using pre-cleaved precursor CYC1 RNA (CYC1pre), previous *in vitro* polyadenylation studies have shown that poly(A) tail lengths are restricted to 50–70 nucleotides using wild-type *PAB1* extracts while *pab1Δ* extracts hyperadenylate CYC1pre (Amrani *et al.*, 1997; Minvielle-Sebastia *et al.*, 1997) (Figure 6A, compare *pab1Δ* and *PAB1* control lanes). Addition of bacterially expressed Pab1p to *pab1Δ* extracts results in shortening of

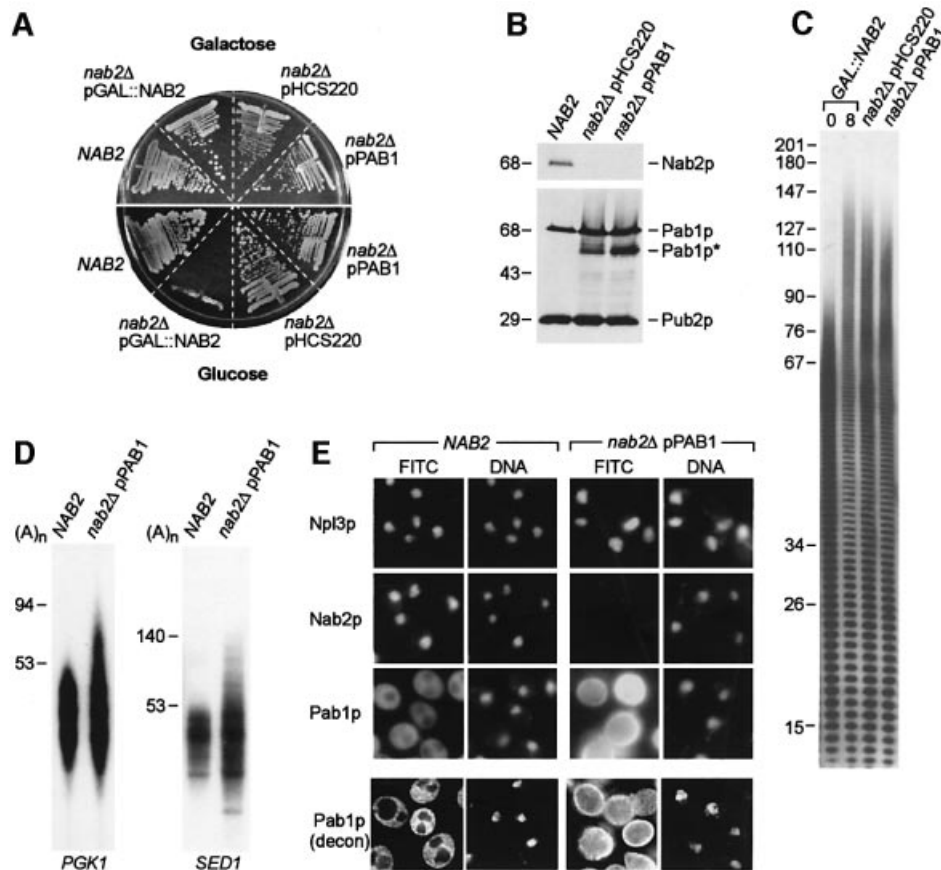


Fig. 4. *PAB1* is a high-copy suppressor of *nab2Δ*. (A) Growth (24°C) of wild-type *NAB2* and *nab2Δ* strains carrying *pHCS220* or *pPAB1*. (B) Immunoblot analysis of Nab2p, Pab1p and Pab1p* expression in *NAB2*, *nab2Δ pHCS220* and *nab2Δ pPAB1* strains. Equal protein loads are shown by immunoblotting with mAb 2B1 (anti-Pub2p). Sizes are indicated in kilodaltons. (C) Poly(A) tail length analyses of *nab2Δ pHCS220* and *nab2Δ pPAB1* strains are compared with hyperadenylated RNAs seen following shift of YJA517-1C (*GAL::NAB2*) cells to glucose for 8 h. (D) Hyperadenylated *PGK1* and *SED1* mRNAs in *nab2Δ pPAB1* cells. Poly(A) tail lengths were determined using LM-PAT. Poly(A) tail lengths are indicated and were determined by comparison with a 100 nucleotide DNA ladder. (E) Pab1p appears in the nucleus when overexpressed in the *nab2Δ* *pPAB1* strain. Panels in the top three rows show localization of Npl3p, Nab2p and Pab1p using conventional immunofluorescence and mAbs 1E4, 3F2 and 1G1, respectively, in *NAB2* and *nab2Δ pPAB1* cells at 24°C. The panels in the bottom row are deconvolution images (Pab1p, decon) showing Pab1p distribution in wild-type *NAB2* versus *nab2Δ pPAB1* cells. Pab1p localization in vacuoles is probably due to increased Pab1p turnover in *nab2Δ pPAB1*.

the poly(A) tail to within the normal 50–70 nucleotide range. A similar effect on polyadenylation was seen when recombinant Nab2p was added to *pab1Δ* extracts with maximum poly(A) tail length restricted to 60–80 nucleotides following addition of ≥ 400 ng of Nab2p (Figure 6A). Recombinant Nab2p also stimulated polyadenylation 2- to 3-fold. Since poly(A) tails were ~ 10 nucleotides shorter when a *PAB1* wild-type extract was used for polyadenylation (Figure 6A, last lane), Pab1p might recruit PAN activity to shorten tails further. The effect of Nab2p on poly(A) tail length control was specific since the addition of another yeast hnRNP, Nab4p, to *pab1Δ* extracts led to enhanced hyperadenylation. Hyperadenylation was also inhibited in the presence of the bovine nuclear poly(A) tail-binding protein PABPN1. However, polyadenylation was also strongly impaired at ≥ 400 ng of PABPN1, possibly because PABPN1 bound to the poly(A) tail was unable to interact productively with the yeast 3'-end processing machinery. Hyperadenylation was also observed when the purified cleavage and polyadenylation factors CF I and CPF were used for CYC1pre-RNA

polyadenylation in the absence of Pab1p (Amrani *et al.*, 1997; Minvielle-Sebastia *et al.*, 1997). Addition of low levels (25–50 ng) of Nab2p increased CYC1pre polyadenylation while limiting tail length to 60–80 nucleotides, while ≥ 100 ng of Nab2p inhibited polyadenylation (Figure 6B). This activity did not require pre-cleaved RNA substrates since Nab2p also enhanced polyadenylation while restricting poly(A) tail lengths during a coupled cleavage–polyadenylation reaction with extracts (Figure 6C), as well as in a reconstituted reaction with purified factors (data not shown).

To discriminate between potential roles for Nab2p in limiting tail length during polyadenylation versus poly(A) tail trimming following synthesis, we examined the time course of polyadenylation in *pab1Δ* extracts following addition of 400 ng of GST–Nab2–His₆. Poly(A) tails in the range of 60–80 nucleotides appeared as soon as 2 min following the initiation of synthesis and, although the conversion of CYC1pre continued up to 180 min, tail length distribution did not change significantly during this period (Figure 6D). We conclude that Nab2p is the

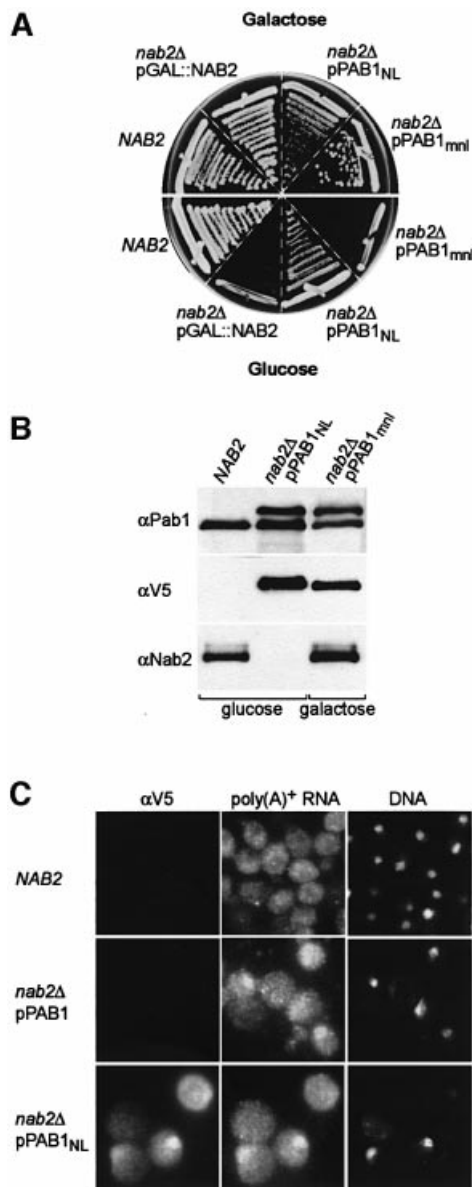


Fig. 5. Targeting of Pab1p to the nucleus suppresses the *nab2Δ* growth defect. (A) Suppression of *nab2Δ* by low-copy pPAB1_{NL}. Plate growth of *NAB2*, *nab2Δ* pGAL::NAB2, *nab2Δ* pPAB1_{NL} and *nab2Δ* pPAB1_{mnl} strains on YPGal (galactose) and YPD (glucose) at 24°C is shown. (B) Immunoblot analysis of *NAB2*, *nab2Δ* pPAB1_{NL} and *nab2Δ* pPAB1_{mnl} strains grown on YPD (glucose) and YPGal (galactose). Proteins were detected using anti-V5 (αV5), anti-Pab1p 1G1 (αPab1p) and anti-Nab2p 3F2 (αNab2p) antibodies. (C) SV40 NLS-tagged Pab1p accumulates in the nucleus. The localizations of V5-tagged Pab1p, poly(A)⁺ RNA and DNA are compared in *NAB2*, *nab2Δ* pPAB1 and *nab2Δ* pPAB1_{NL} strains.

primary factor that controls poly(A) tail length during mRNA 3'-end formation, which is consistent with its predominantly nuclear localization.

Discussion

Nab2p is required for poly(A) tail length control during mRNA 3'-end formation

In this report, we demonstrate that the yeast hnRNP Nab2p is required for mRNA poly(A) tail length control *in vivo*

and *in vitro*. Evidence that Nab2p plays an essential and direct role in limiting tail length during polyadenylation includes: (i) Nab2p depletion *in vivo* leads to the appearance of hyperadenylated mRNA; (ii) unlike mutations in mRNA export factors, long poly(A) tails are not the indirect result of a block to mRNA export since hyperadenylated mRNA is present in *nab2-21* with a normal subcellular distribution of poly(A)⁺ RNA; (iii) either *PAB1* overexpression, or expression of nuclear-targeted Pab1p, fails to correct the hyperadenylation phenotype of *nab2* mutant strains; (iv) Nab2p stimulates polyadenylation while inhibiting hyperadenylation *in vitro* in the absence of Pab1p; and (v) in contrast to Pab1p, Nab2p does not show poly(A) tail trimming activity *in vitro*. Why have previous biochemical fractionation studies failed to uncover Nab2p as a factor that regulates polyadenylation *in vitro*? First, these studies employed whole-cell extracts that contain relatively high Pab1p levels, and we show here that Pab1p masks the polyadenylation function of Nab2p *in vitro*. Secondly, Nab2p in these extracts is bound to Kap104p and this association probably interferes with Nab2p-RNA interactions (Lee and Aitchison, 1999).

Previous studies have suggested that Pab1p is an important 3'-end processing factor *in vivo* since it is required for polyadenylation *in vitro*, co-purifies with CF I and interacts directly with Rna15p, which is an essential factor for polyadenylation (Amrani *et al.*, 1997; Minvielle-Sebastia *et al.*, 1997). Interestingly, *PAB1* is also a multicopy suppressor of *rna15-2* (Amrani *et al.*, 1997). While *NAB2* expression is essential for normal polyadenylation *in vivo*, our results do not exclude a role for Pab1p in mRNA 3'-end formation or the possibility that Nab2p and Pab1p are both associated with the poly(A) tail in the nucleus. An alternative possibility is that Nab2p binds to A-rich tracts within specific pre-mRNAs and the extent of polyadenylation is regulated by Nab2p interactions with Pab1p bound to the poly(A) tail.

Based on previous studies and the work reported here, we propose that poly(A) tails are synthesized to a default length in the presence of Nab2p, and that Pab1p recruits PAN activity for message-specific trimming of the poly(A) tail. According to this proposal, Nab2p is recruited initially to the mRNA 3'-end, possibly by the growing poly(A) tail. What is the evidence that the poly(A) tail is important for Nab2p function? This hnRNP contains an unusual RNA-binding motif consisting of seven C₃H repeats, which is required for poly(A)-binding activity *in vitro* (Anderson *et al.*, 1993b). Deletion of two of these repeats in the *nab2-21* allele results in mRNA hyperadenylation, suggesting that poly(A) binding is compromised in this mutant. In addition, Pab1p masks the hyperadenylation inhibitory function of Nab2p *in vitro*. Since these two proteins are not structurally related, but both bind to poly(A), it is likely that Pab1p blocks Nab2p activity *in vitro* by sequestration of the poly(A) tail. This is consistent with our observation that another unrelated poly(A)-binding protein, PABPN1, also inhibits polyadenylation *in vitro*. The presence of excess Pab1p in cell extracts might limit accessibility to the poly(A) tail and also prevent hyperadenylation by simultaneous recruitment of PAN activity. Following tail binding, we suggest that Nab2p interacts with additional 3'-end processing

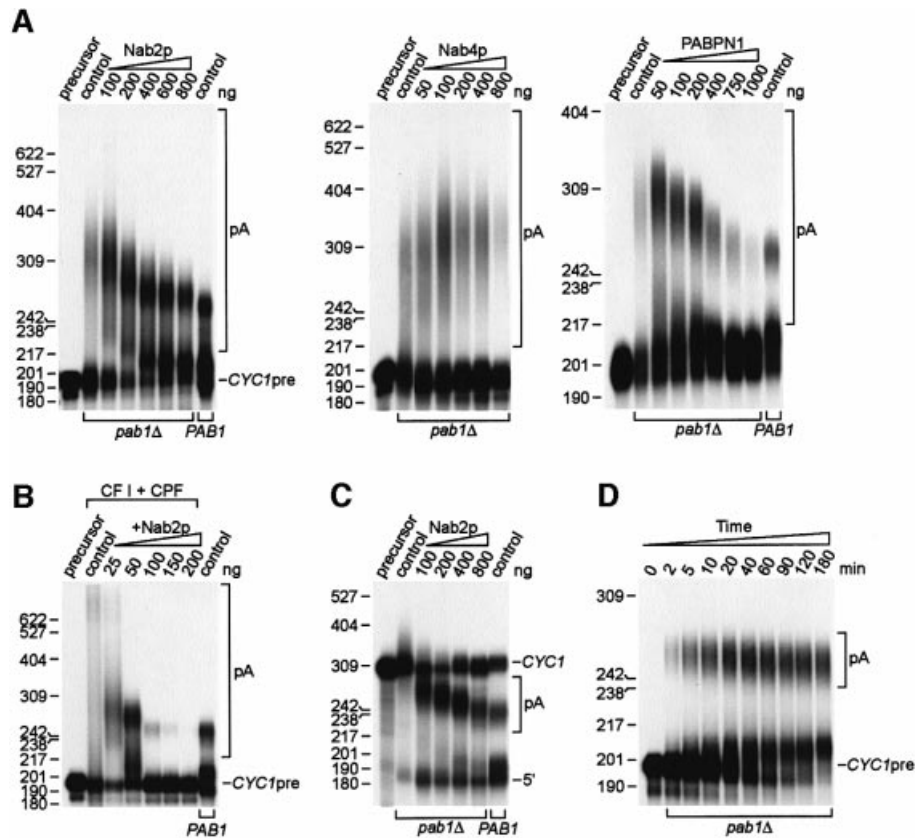


Fig. 6. Nab2p is required for poly(A) tail length control during polyadenylation *in vitro* in the absence of Pab1p. (A) Recombinant Nab2p stimulates polyadenylation but inhibits hyperadenylation *in vitro*. Reactions were performed using pre-cleaved CYC1 precursor RNA (CYC1pre) and either YAS394 (*pab1Δ*) or wild-type (*PAB1*) cell extracts. Extracts were either used directly (control lanes) or supplemented with 100–800 ng of GST–Nab2p–His₆ (left panel), 50–800 ng of GST–Nab4p (middle panel) or 50–1000 ng of purified bovine PABPN1 (right panel). Polyadenylated RNAs are bracketed (pA). The indicated size markers are pBR322 *Msp*I fragments. (B) Hyperadenylation is inhibited by Nab2p during polyadenylation *in vitro* using purified cleavage and polyadenylation factors. Reactions were performed using CYC1pre, CF I + CPF, 10 ng of GST–Nab4p and 25–200 ng of GST–Nab2p–His₆. The last lane is a control reaction using wild-type *PAB1* extract. (C) Nab2p controls poly(A) tail length during coupled cleavage–polyadenylation *in vitro*. Reactions were performed using CYC1 RNA instead of pre-cleaved precursor. The positions of the CYC1 RNA (CYC1), polyadenylated CYC1 (pA) and the 5' cleavage fragment (5') are indicated. (D) Time course analysis showing that Nab2p restricts poly(A) tail length to 60–80 nucleotides during polyadenylation and does not promote tail trimming.

factor(s), possibly Nab4p, since these two hnRNPs interact in the two-hybrid system (Uetz *et al.*, 2000), resulting in the termination of polyadenylation.

Potential roles for Nab2p, Pab1p and the poly(A) tail in mRNA export

Considerable evidence supports the proposal that mRNA 3'-end processing and nuclear export are tightly coupled *in vivo* (Brodsky and Silver, 2000; Hilleren *et al.*, 2001; Jensen *et al.*, 2001). When temperature-sensitive *dbp5/rat7*, *gle1/brr3*, *mex67*, *mtr2*, *nup159/rat8p* and *rip1* strains are shifted to restrictive growth temperatures, mRNA export is blocked and hyperadenylated mRNA accumulates in the nucleus. Since long poly(A) tails are not detectable at permissive growth temperatures in these export mutants, inhibition of mRNA export may lead to the titration of a factor that is required for the termination of polyadenylation (Hilleren and Parker, 2001; Jensen *et al.*, 2001).

Although we have provided evidence that Nab2p is a polyadenylation termination factor, perhaps the most surprising result of our studies is that hyperadenylation

can occur in the absence of a detectable mRNA export defect in *nab2-21* cells. Of course, polyadenylation control might be impaired, but not completely compromised, in *nab2-21* at 30°C, and thus transcripts are released at rates compatible with normal mRNA export. An important caveat is that the poly(A) tail length and poly(A)⁺ RNA localization assays are probably not equally sensitive so there may be an undetectable export problem in *nab2-21* at 30°C. Another possibility is that Nab2p is required independently for the regulation of mRNA polyadenylation and nuclear export. For mRNA export, Nab2p might associate with transcripts during 3'-end formation and interact subsequently with an NPC-associated factor to form a complex that promotes mRNP targeting to the NPC, similar to the function of the Mex67p–Mtr2p complex for transcribed regions of the mRNA (for reviews see Cole, 2000; Cullen, 2000). In support of this idea, Nab2p shuttles between the nucleus and cytoplasm and interacts *in vivo* with the NPC-associated protein, Gfd1p (Hodge *et al.*, 1999; Strahm *et al.*, 1999; Duncan *et al.*, 2000). An alternative model is that Nab2p association with the nascent transcript antagonizes the activity of the

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PABPN1 (kind gift of Elmar Wahle, University of Halle-Wittenberg, Halle Saale, Germany). For the polyadenylation time course, a 12-fold reaction mixture containing YAS394 extract was supplemented with 9.6 µg of GST-Nab2p-His₆ and pre-incubated at 30°C for 15 min in the absence of the substrate RNA. Polyadenylation was initiated by addition of labeled transcript, aliquots of 20 µl were withdrawn at the times indicated, and reactions were stopped and analyzed on 40 cm long 6% polyacrylamide–8.3 M urea gels.

For preparation of full-length recombinant Nab2p, a His₆ tag was cloned in-frame at the 3' end of the NAB2 open reading frame. The NAB2 gene was amplified from L4717 genomic DNA using primers MSS1131 (5'-GCGGAATTCATGTCTCAAG-AACAGTACACAGAAAAC-3') and MSS1132 (5'-GCGGTCGACTCAGCCGCTGCTGTGATGATGATGATGATGGCTGCTGCCGTTTCATTTCCGTATCTTGTCTTGT-3'), and the PCR product cloned into pGEX-4T-1 (Amersham Pharmacia Biotech) between the *EcoRI* and *SalI* sites. The resulting GST-Nab2p-His₆ protein was expressed in BL21-CodonPlus (DE3) cells (Stratagene) and purified using His-Bind (Novagen, Madison, WI) and glutathione–Sepharose (Amersham Pharmacia Biotech) chromatography.

Acknowledgements

We thank J.Aris, A.de Bruyn Kops, S.Butler, T.-H.Chang, E.Hurt, C.Guthrie, A.Sachs, K.Sträßer and E.Wahle for strains, reagents and protocols. We also thank M.Paddy for help with deconvolution microscopy, and J.Aris, S.Butler and A.Lewin for comments on the manuscript. This work was supported by NIH grant GM46272 (M.S.S.), a pre-doctoral trainee award to R.E.H. (NIH T32-AI-07110), and by the CNRS, the Ministère de la Recherche Scientifique, La Fondation pour la Recherche Médicale/Fondation BNP-Paribas and La Ligue Nationale contre le Cancer (L.M.-S.). S.D. was the recipient of a post-doctoral fellowship from La Ligue Nationale contre le Cancer.

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*Received June 29, 2001; revised February 5, 2002;
accepted February 7, 2002*